

The Promotion of Spore Germination and Gametophyte Development in *Ophioglossum palmatum* by Low pH

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Gametophytes of several terrestrial species of the Ophioglossaceae have been grown from spores in axenic culture (Whittier, 1972, 1981, 1983; Gifford & Brandon, 1978; Whittier & Peterson, 1984). As more species have been included in these investigations, it has become evident that one set of cultural conditions is not effective for all species. For the species examined to date, darkness is needed for spore germination and sugar is necessary for growth of these nonphotosynthetic gametophytes. However, variations in the mineral content, nitrogen source, and temperature are needed to promote or allow gametophyte formation from spores in some species (Whittier, 1981; Melan & Whittier, 1990).

Since variation exists in the requirements for gametophyte development in culture for the terrestrial species, an examination of the conditions necessary for gametophyte development of an epiphytic species was undertaken. It seemed appropriate to determine if there was anything unusual about the conditions that are necessary to obtain gametophytes of epiphytic species from spores. *Ophioglossum palmatum*, an epiphyte native to the United States, was selected for testing. In Florida, this *Ophioglossum* typically grows on cabbage palms (Mesler, 1975), however there is one report of it growing on saw palmettos (Nauman & Moyroud, 1986). The objectives of this study were to determine the requirements for spore germination, the pattern of early gametophyte development, and the possibility of raising mature gametophytes in axenic culture.

MATERIALS AND METHODS

Spores of *Ophioglossum palmatum* L. were obtained from fertile spikes collected in southern Florida. Since *O. palmatum* is an endangered species in Florida, only the fertile spikes were collected and no plants or leaves were removed for voucher specimens. The spores had the typical shape and internal organization for *Ophioglossum* spores and very few atypical spores with irregular shapes or disorganized contents were present in any of the spore collections.

The spores were surface sterilized with 20% Clorox by the technique of Whittier (1973). The surface-sterilized spores were sown in the early experiments on 15 ml of nutrient medium in 20 mm diameter culture tubes, which were tightly closed to reduce moisture loss. Except for a few light controls, the spores were maintained in darkness at 20±1°C for 3 to 12 months. No germination occurred in any of the cultures in the light.

The nutrient medium contained a modified Moore's solution (Moore, 1903). A liter of the basic medium contained 100 mg MgSO₄·7H₂O, 40 mg CaCl₂, 100 mg K₂HPO₄, and 100 mg NH₄Cl, plus FeEDTA and minor elements. The carbon source was 0.2% glucose. The media employed in the early experiments were solidified with 0.6% agar and were pH 5.0 or 5.5 after autoclaving. Other aspects of the nutrient medium and culture

vessels will be considered with the results since they were important for spore germination and gametophyte growth.

RESULTS

The early experiments had limited success. A low percentage of germination occurred on the pH 5.0 medium after three months. However, the young gametophytes aborted at the 2- or 3-celled stage. Efforts to modify the nutrient medium involved alterations to the pH. A wider pH range was employed with these media and after nine months, there was good germination on the more acidic media (Table 1). The gametophyte growth was again limited and rarely exceeded the 4-celled stage.

After several futile attempts were made to improve the growth of the gametophytes on these media, the natural habitat of the gametophyte was analyzed more closely. In nature these gametophytes grow in the black humus which collects between the petiole bases and trunks of *Sabal palmetto* (Walt.) Lodd. ex Schultes. Mesler (1975) has found that only the humus which remains moist will have gametophytes and Wherry (1964) reported that the pH of this material is subacid. The nutrient media employed would seem to satisfy both conditions. They were moist and at pH 5.0 or 5.5 which fits Wherry's pH scale (1920) as being subacid.

Table 1. Spore germination of *Ophioglossum palmatum*¹

Medium and duration	pH							
	3.4	4.0	4.2	4.6	4.7	5.0	5.4	6.2
Agar, 9 mo.	-	-	-	48%	-	23%	0%	0%
Agar, 3 mo.	-	16%	5%	-	-	-	-	-
Liquid, 8 mo.	14%	55%*	-	-	48%*	-	20%	-

¹Percentage of germination followed by an asterisk (*) indicates media which supported gametophyte growth beyond the 4-celled stage.

Poor growth indicated that the nutrient medium did not satisfy all of the requirements of these gametophytes. Since there were some indications that the pH of the humus might be more acidic than assumed, its pH was tested. The humus is strongly acid at pH 4.0+0.1 which suggested that the pH of the nutrient medium should be changed.

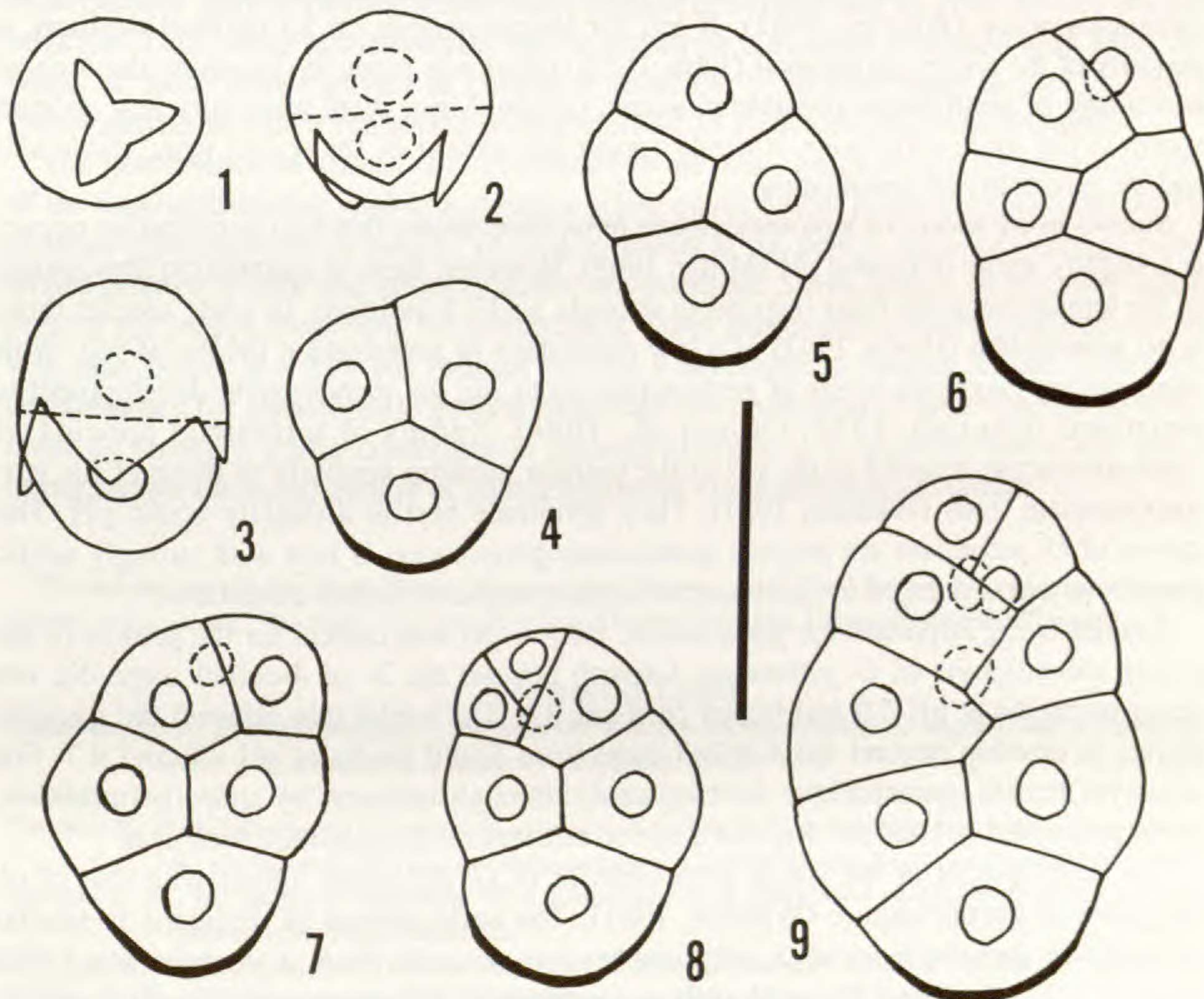
There are difficulties in solidifying agar of very acidic nutrient media. However, increasing the concentration of agar did alleviate the problem to some extent. After three months on a medium with 1.5% agar and at pH 4.0 after autoclaving, 16% of the spores germinated (Table 1). Germination on a medium at pH 4.2 was 5%.

In an effort to better control the initial pH of the nutrient medium, shallow liquid culture in plastic culture flasks was tested. The nutrient media were filter sterilized and had initial pH readings of 3.4, 4.0, 4.7, and 5.4. Spores germinated on all media and the higher percentages of germination, 55% and 48%, occurred on media with pH 4.0 and 4.7 respectively (Table 1). Importantly, the media at pH 4.0 and 4.7 supported gametophyte growth with the best being at pH 4.0.

Cracking open of the triradiate ridge was the first indication that germination had started (Fig. 1). Before there was any significant enlargement of the spore, a division perpendicular to the polar axis of the spore occurred (Fig. 2). It divided the spore equally forming a distal cell (away from the triradiate ridge) and a proximal cell (near the triradiate ridge). The proximal cell enlarged forcing the three lobes of the cracked spore coat apart

and bulged out of the spore coat (Fig. 3). The outer wall of the proximal cell thickened as the gametophyte developed but mucilage did not appear to be secreted through this thick wall (Fig. 4). The second division, which was slightly oblique to the polar axis of the spore, divided the distal cell into two cells with one slightly larger than the other (Fig. 4). The third division, which occurred in the larger of these cells (Fig. 5), was initially perpendicular to the cell wall of the second division. The 4-celled gametophyte was a flat plate of cells. The fourth division occurred in the terminal cell and the gametophyte began to thicken (Fig. 6). Additional divisions in the terminal cell established an apical cell (Fig. 7, 8, 9). Although there was some variability in the stage at which the apical cell was established, the apical cell was usually present by the 8- or 9-celled stage. The proximal cell did not divide during the early stages of gametophyte development. Whether it underwent divisions at later stages was not determined.

Gametophytes were transferred from the pH 4.0 and 4.7 liquid media to new media in an effort to obtain mature gametophytes. Even though these cultures were rather old at the time of transfer and most of the gametophytes were of microscopic size, it was the



Figs. 1-9. Young gametophytes of *Ophioglossum palmatum*. Fig. 1. Opening of triradiate ridge. Fig. 2. Early two-celled gametophyte. Cell wall and nuclei covered by spore coat indicated by dotted lines. Fig. 3. Two-celled gametophyte with proximal cell bulging out of the spore coat. Fig. 4. Three-celled gametophyte with thickened proximal cell wall. Figs. 5-8. Four-, five-, six-, and seven-celled gametophytes respectively. No effort was made to indicate walls of cells positioned behind other cells, however the nuclei of these cells are indicated by dotted lines. Fig. 9. Ten-celled gametophyte with apical cell. Bar = 100 μ m.

only source of gametophytes for new cultures. They were transferred to a new medium with 1.0% agar and at pH 4.4 after autoclaving. The microscopic-sized gametophytes were pipeted from the liquid media to the new medium. The macroscopic-sized gametophytes were moved individually with a spatula to the new medium. The microscopic-sized gametophytes ceased to grow after the transfer, however the macroscopic gametophytes grew very slowly on the new medium. They first became cylindrical and later began to branch.

DISCUSSION AND CONCLUSIONS

Nutrient media with ammonium-nitrogen are very effective for the germination of *Botrychium* spores (Melan & Whittier, 1990). This is also true for spores of *O. palmatum* but the pH of the medium must be lowered to get high percentages of germination. The most germination was obtained on a medium at pH 4.0, although germination did occur on media with a somewhat higher or lower pH.

The initial germination of *O. palmatum* spores occurred at about 3 months in the dark, which is within the range of times necessary for the germination of other spores of the Ophioglossaceae (Whittier, 1981). If left for longer periods on an optimal medium, a majority of the spores germinated (Table 1). No effort was made to determine the highest percentage of germination possible in axenic culture. Since there were very few aborted spores in the spore collections, it would appear that almost all the spores were viable and had the possibility of germinating.

Studies using spores of leptosporangiate ferns have shown that best germination occurs at a slightly acidic or neutral pH (Miller, 1968). However, there is variation in how spores of the leptosporangiate ferns respond to strongly acidic conditions. In some species there is no germination (Hevly, 1963) or a low percentage of germination (Mohr, 1956). With other species moderate levels of germination occur but the gametophyte development is restricted (Courbet, 1955; Otto et al., 1984). Spores of terrestrial species of Ophioglossaceae respond to the pH of the nutrient medium similarly to those of the leptosporangiate ferns (Whittier, 1981). They germinate best at a slightly acidic pH. The spores of *O. palmatum* are unusual in that their germination is best with strongly acidic conditions and is reduced or eliminated at slightly acidic or neutral conditions.

Besides being important for germination, the low pH was critical for the growth of the young gametophyte of *O. palmatum*. Growth beyond the 3- or 4-celled stage did not occur on media at pH 5.0 and higher or at pH 3.4. The media that allowed the gametophytes to develop beyond the 4-celled stage were liquid media at pH 4.0 and 4.7. For whatever reason, gametophyte development, although initiated by spore germination, never proceeded beyond the 4-celled stage on nutrient media solidified with agar.

The development of the young gametophytes of *O. palmatum* agrees with that of *O. vulgatum* in axenic culture (Whittier, 1981). The early pattern of divisions is similar although the gametophytes of *O. vulgatum* become two cells thick at an earlier stage than those of *O. palmatum*. The large cells and somewhat different pattern of cell divisions make the *Ophioglossum* gametophytes distinctive from the young gametophytes of *Botrychium* (Whittier, 1981). The formation of an apical cell by the young gametophytes of *Ophioglossum* also distinguishes these gametophytes from those of *Botrychium* which do not form apical cells.

The development of the gametophyte and its apical meristem from products of the dis-

tal cell is characteristic of the Ophioglossaceae. This pattern is not common among the other pteridophytes. It has only been found in *Actinostachys pennula* for pteridophytes with mycorrhizal gametophytes (Bierhorst, 1975). Spores of leptosporangiate ferns have several germination patterns, however, in only two of these does the proximal cell become a rhizoid and the distal cell develop into the photosynthetic gametophyte (Nayar and Kaur, 1971). Under natural conditions, there may be a possible advantage for the Ophioglossaceae to produce the growing portion of the gametophyte from the distal cell. Campbell (1911) has reported that the mycorrhizal infection of the young gametophytes of *O. pendulum* occurs through the wall of the proximal cell. With this germination pattern the proximal cell, which is first out of the spore coat and becomes a reduced rhizoid, can be infected by a mycorrhizal fungus during the early states of germination without interfering with the meristematic portion of the young gametophyte.

Microscopic multicellular gametophytes failed to grow into cylindrical gametophytes when transferred to an agar medium at pH 4.4. Whether this was a problem with the medium or the age of the young gametophytes has not been resolved at this time. Macroscopic gametophytes which were transferred continued to grow slowly on this medium. Thus, older gametophytes can be grown on a medium solidified with agar. The shape of these gametophytes in culture is sufficient to identify them as *Ophioglossum* gametophytes and their branching is characteristic of gametophytes of *O. palmatum*.

The germination of spores of *O. palmatum* in the dark, which is true for other members of the Ophioglossaceae, and in a strongly acidic environment helps to explain the habitat of these gametophytes in nature. The black humus at the petiole bases of cabbage palms would provide a dark and strongly acidic environment. Moist humus of this type would present the proper conditions for germination. Since gametophyte development in culture was also better at a low pH, humus having a mycorrhizal fungus would satisfy the requirements for gametophyte growth. Although the promotion of gametophyte development by strongly acidic conditions is unusual in ferns, it is apparent that a low pH is important for the development of gametophytes of *Ophioglossum palmatum*.

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REVIEW

New Jersey Ferns and Fern-Allies by James D. Montgomery and David E. Fairbrothers. Rutgers University Press, New Brunswick, NJ 08901. ISBN 0-8135-1817-2, Cloth \$45.00. 292 pp.

This is an excellent and comprehensive treatment of the 83 species plus 32 hybrid pteridiophytes found in New Jersey. The authors have written a very useful manual. The descriptions are accurate, brief, and clearly written. The keys are carefully crafted to focus on distinctions to fertile specimens and to vegetative specimens. The detailed illustrations of whole fronds and enlargements of diagnostic features, prepared by Kathleen L. John-Alder, are excellent. The treatments include comments on taxonomic status, habitat, chromosome counts, habit of growth, endangered species status, and distribution maps. The size of dot varies, with larger dots indicating older collections, allowing the reader to infer spread or depletion of a species. Access to the book is facilitated with illustrations of technical terminology, with discussions of the biology of hybridization, and with an explanation of the physiographic regions and habitats of New Jersey. The bibliography is extensive, providing ample means to consult the literature. Interestingly, the ferners who collected and investigated ferns in New Jersey over the last 250 years include many eminent botanists, including Bartram, Kalm, Michaux, Pursh, Barton, Nuttall, Torrey, Britton, and Stone. This manual is useful to amateur and expert alike. — JAMES H. PECK, Department of Biology, University of Arkansas at Little Rock, Little Rock, AR 72204.